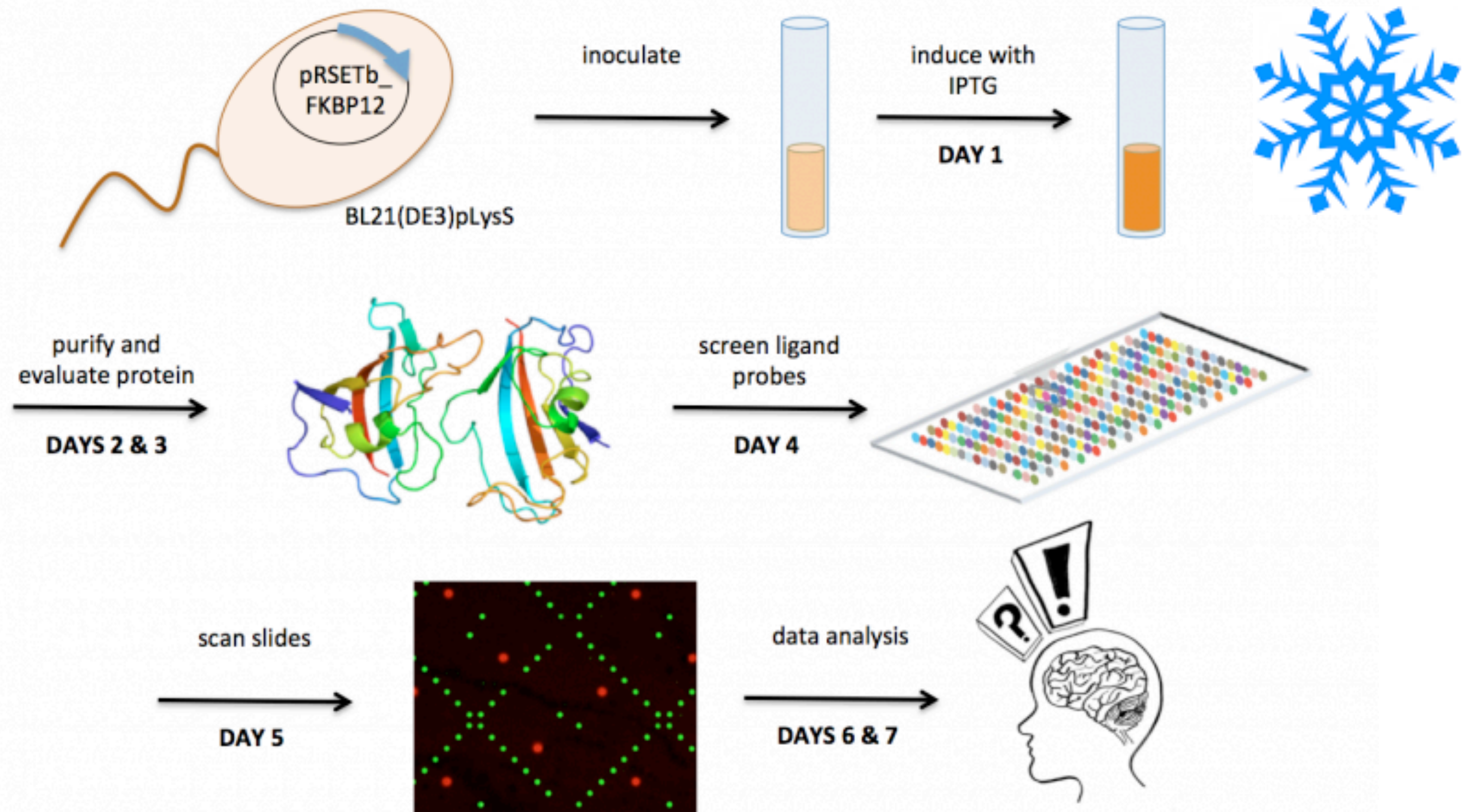
M1D2:
Purify induced protein

02/14/2017

Happy Valentine's Day!

MIT

Overview of
“M1: High-throughput ligand screening”



M1 major assignments

- Data summary (15%)
  - in teams, on Stellar
  - draft due 03/10, final revision due 03/27
  - bullet points, .PPTX
- Mini-presentation (5%)
  - individual, video via Gmail
  - due 03/18
- Lab quizzes (extra credit on homework grade)
  - M1D3, M1D5, and M1D7
- Notebook (5% total)
  - one day will be collected and graded by Rob on M1D7
- Blog: http://be20109s17.blogspot.com/ (participation: 5% total)
  - by 04/03

Office hours

Noreen Lyell

- M 2-5
- in 16-317

Leslie McClain

- T 9:30-11
- in 56-341c

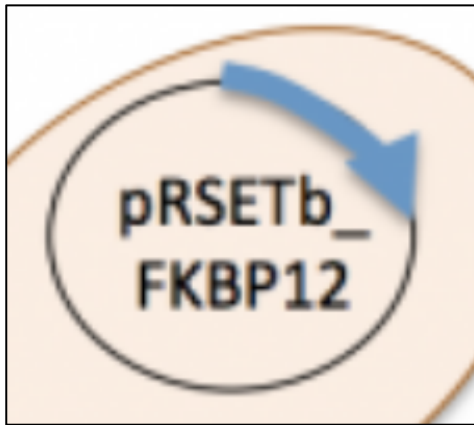
Maxine Jonas

- R 9:30-11
- in 16-239

by appointment: nllyell@, lesliemm@, jonas_m@

don't hesitate!

What you worked on, on snowy M1D1



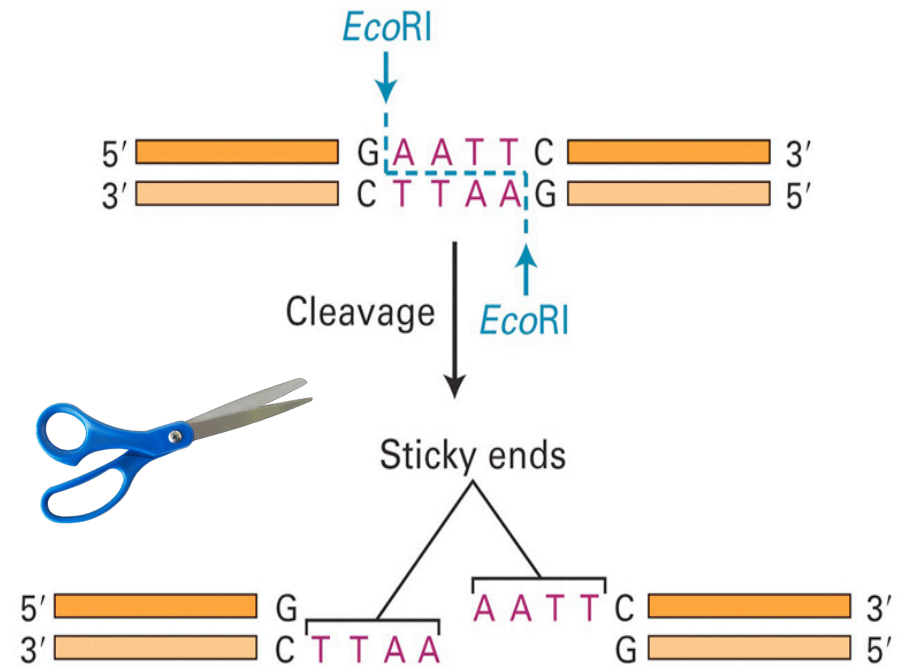
- Reproduce *in silico* the cloning of pRSETb-FKBP12
  - *A Plasmid Editor* (APE)
  - PCR amplification of *Fkbp12* **insert**
  - digestion of pRSETb **vector** by endonucleases
  - ligation

Questions?

Restriction enzymes

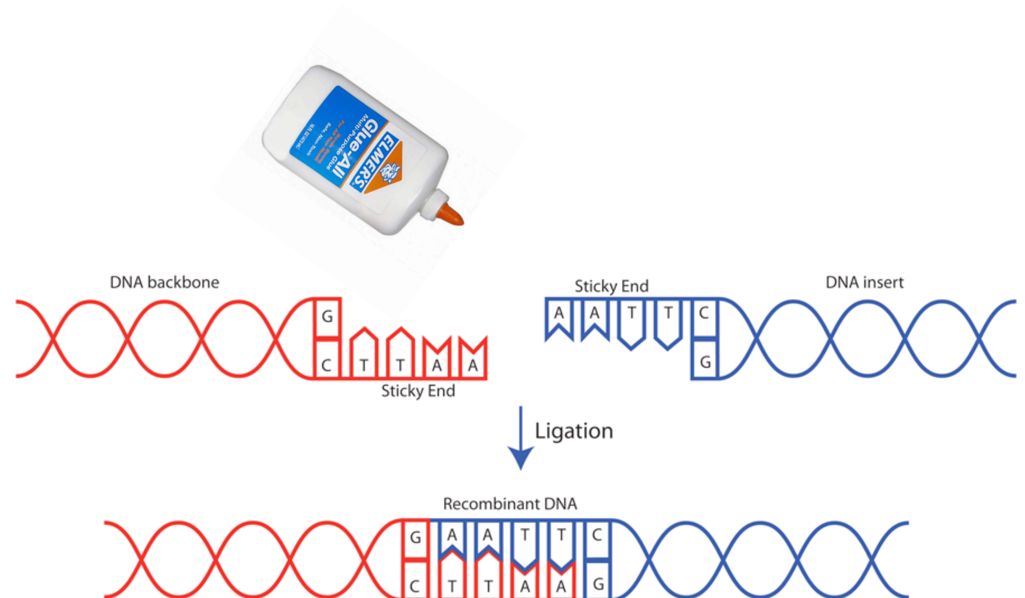
- homodimers
- cut at palindromic recognition sites
- recommended: 5-10 U per µg of DNA

role of "junk" at the end of flap?



Ligase

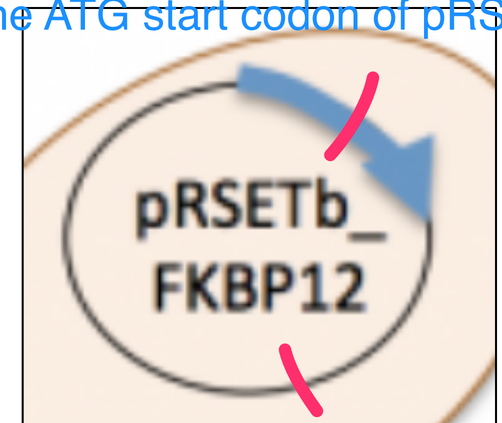
- forms covalent phosphodiester bond between 3’ OH acceptor and 5’ phosphate donor
- requires ATP



My questions for you, about M1D1:

- Did your primers meet all ideal criteria (length, GC content, T_m, …)?
- What are BamHI and EcoRI?
- What was the size of your PCR product? ~ 341 bp
- How about after digestion with BamHI and EcoRI? 331 bp
- What did the sequence of your reverse primer look like?
  - end of *Fkbp12* gene + EcoRI site + junk
  - 5' junk + EcoRI site + end of *Fkbp12* gene 3'
- Why did you add a T? puts the gene in frame with the ATG start codon of pRSETb

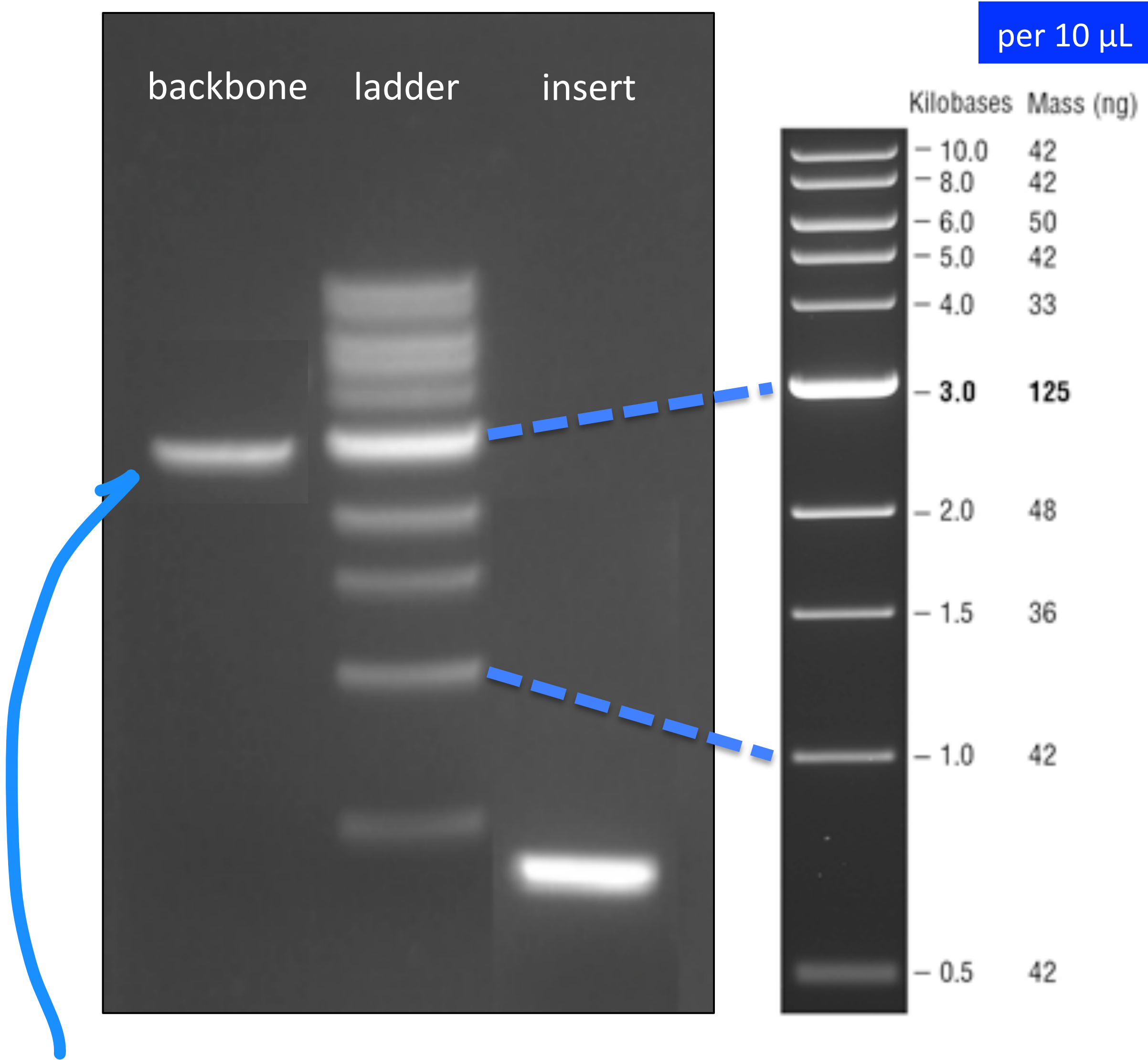
  A
- For your confirmation digest,
  - Which 2 endonucleases did you pick?
  - Which NEB buffer do they work best in?
  - What size fragments did you anticipate?



AvaI + BlpI : cut at 317 & 587

- EamII05I + SmaI : 1380 & 1800

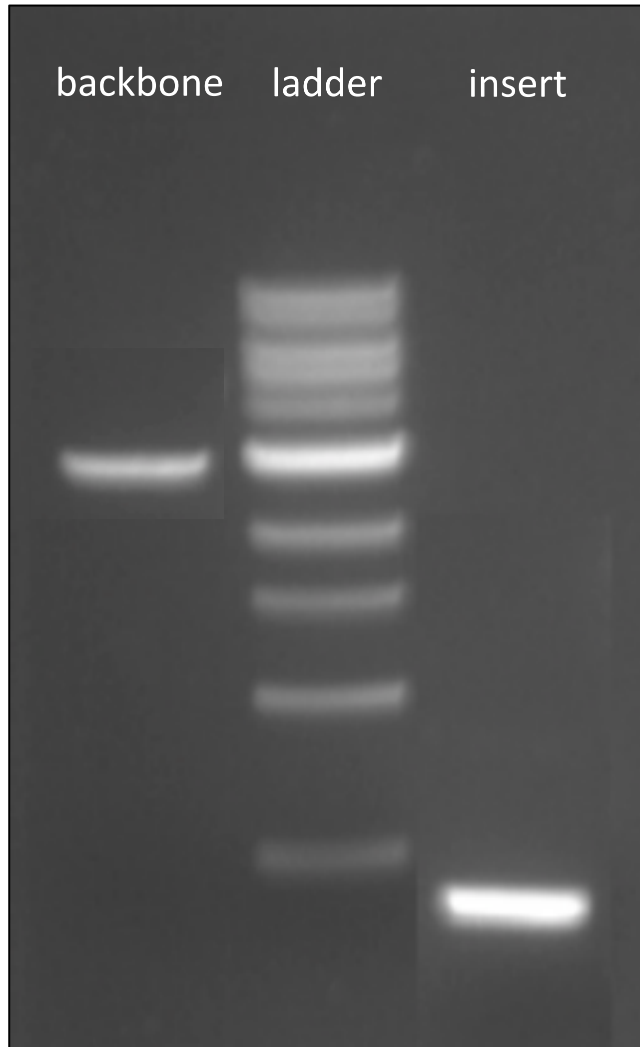
For ligation, mix 1:4 *molar* backbone : insert



- Assuming
  - 10 µL of ladder loaded,
  - 5 µL of *BamHI-EcoRI* double digest loaded,
- amount of backbone =
  100 ng
- amount of insert =
  400 ng

➢ but mass of DNA ≠ molar amount of DNA

Calculate the 1:4 *molar* amounts for ligation



1. From recovery gel, estimate
   - backbone: 100 ng / 5 µL = 20 ng/µL
   - insert: 200 ng / 5 µL = 40 ng/µL

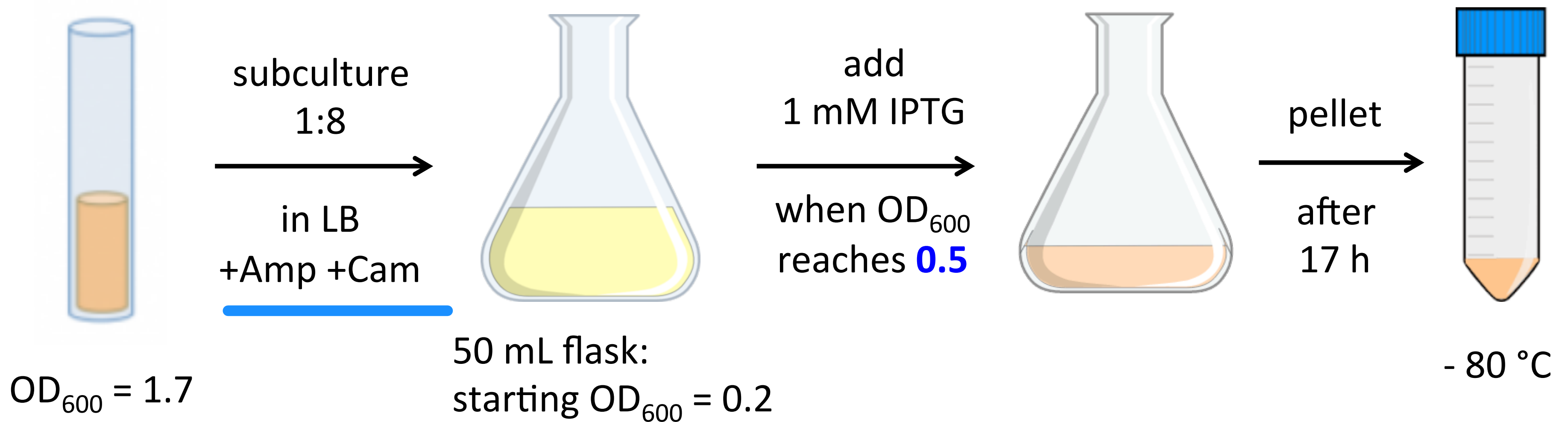
2. Determine volume of backbone needed
   - 50-100 ng, choose 60 ng, *i.e.* 3 µL

3. Calculate moles of backbone
   - **2853 bp** * (660 g / (mol*bp)) = 1.88×10^{6} g/mol
   - so 60 ng / (1.88×10^{6} g/mol) = 3.19×10^{-14} mol

4. Determine moles of insert needed (4X bkbn)
   - $4 \times 3.27 \times 10^{-14}$ ~ 1.27×10^{-13} mol
   - with **341 bp** * (660 g / (mol*bp)) = 2.25×10^{5} g/mol
   - so use 1.27×10^{-13} mol * 2.25×10^{5} g/mol ~ 28.6 ng

5. Calculate volume of insert needed
   - 28.6 ng / (40 ng/µL) ~ 0.7 µL

What happened behind the scene on M1D1



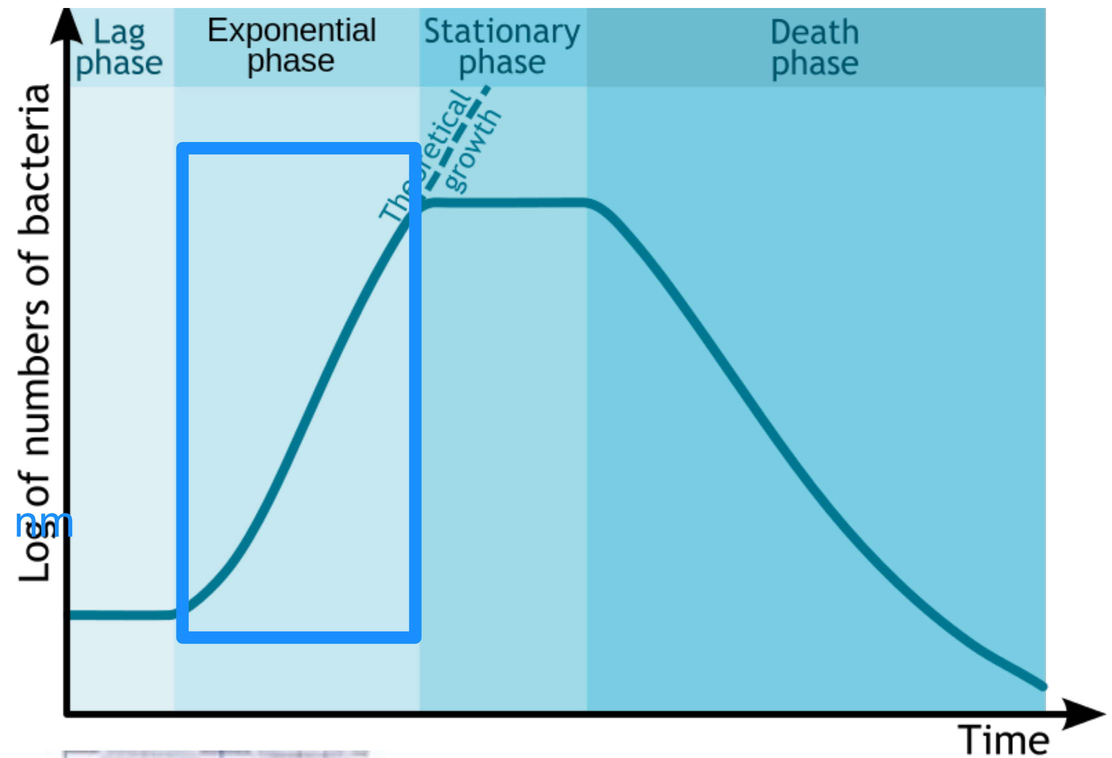
- Induce expression of FKBP12 protein
  - in BL21(DE3)pLysS *E. coli* cells
  - using IPTG

E. coli produces proteins in *growth* phase

- exponential phase
  - binary fission
  - OD_{600} ~ 0.4 - 0.8
  - machinery ready

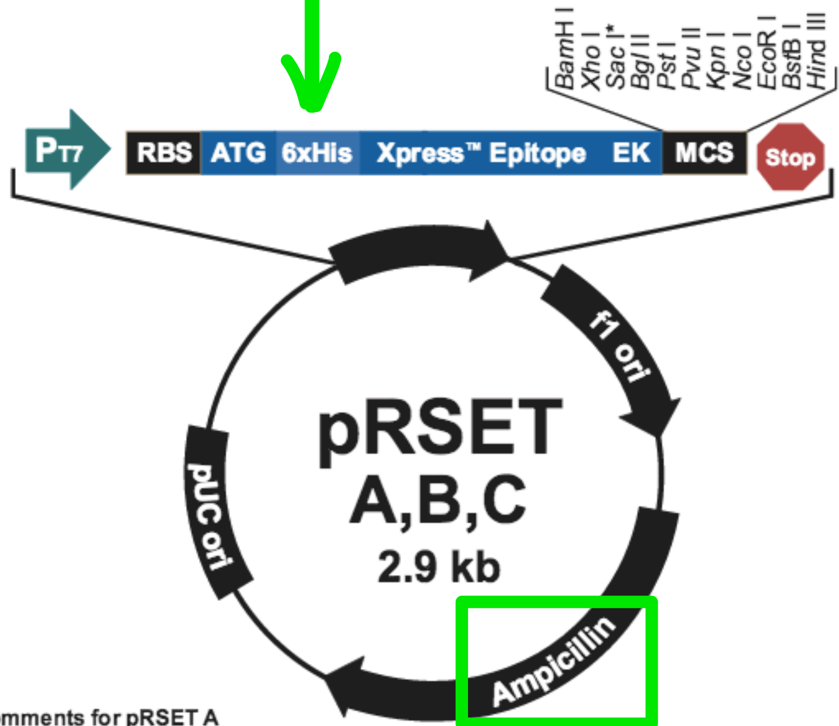
optical density

- OD ≠ absorbance
  - optical density
  - turbidity, scattering of 600 nm
  - cells are yellow,
  - they don't absorb 600 nm
    UV would damage cells



His tag

Let’s take a closer look at the pRSETb vector



*Version C does not contain *Sac* I

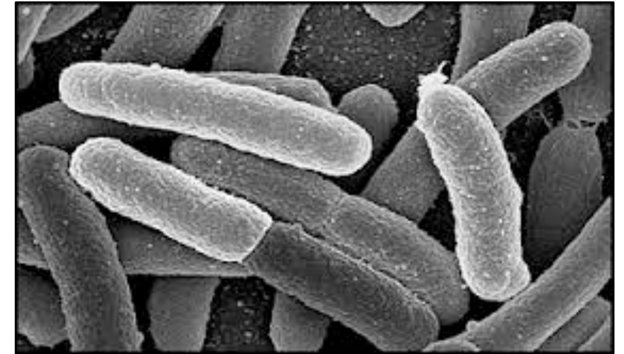
Comments for pRSET A
2897 nucleotides

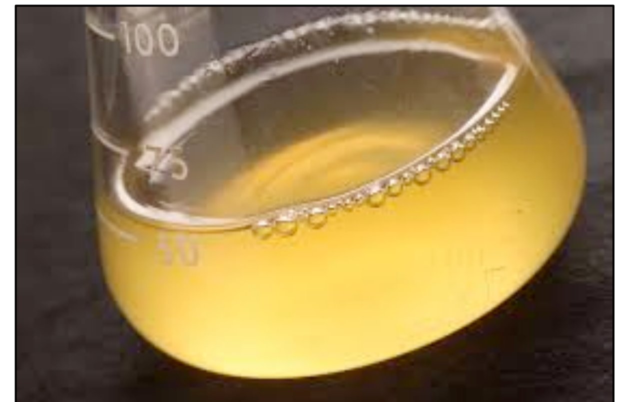
T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 2047-2720 (C)

- P_{T7} — promoter
- RBS — ribosomal binding site recruits ribosome to mRNA, to initiate translation
- ATG — start codon
- 6xHis
- Xpress epitope — antibody recognition possible
- EK (enterokinase) — cleavage site
- MCS — multiple cloning site with many endonuclease recognition sites
- Stop

BL21(DE3)pLysS competent cells

- BL21: E. coli bacterial strain
- can express FKBP12 protein
  - induction by lactose or analog: isopropyl β-D-thiogalactoside (**ITPG**)
  - under ___T7___ promoter control in pRSETb vector
- DE3: bacteriophage (virus)
  - used to integrate the *lac*/T7RNAP construct into *E. coli*
- pLysS: protein that produces
  - lysosyme, which binds to T7RNAP, reducing basal "leaky" expression
  - retained by chloramphenicol (Cam) selection

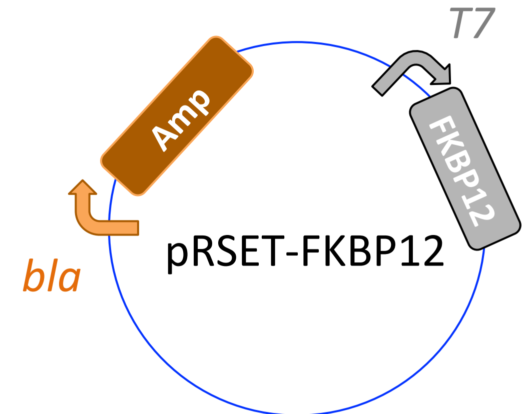




Let's piece together this "protein induction" story

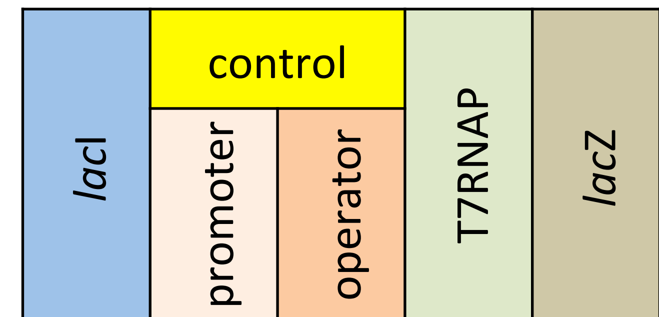
① in the pRSET plasmid

- bla promoter is constitutively *on*
- T7 promoter is turned *on* in the presence of T7 RNA polymerase



② in BL21(DE3)pLysS

- T7RNAP gene engineered in DE3 cells under a modified *lac* operon control
- *lac*I encodes a __repressor______ that binds to _control area______ , thereby turning it *off*
- in addition, T7 lysosyme inactivates T7 promoter



genes of the lac operon

Let's piece together this "protein induction" story

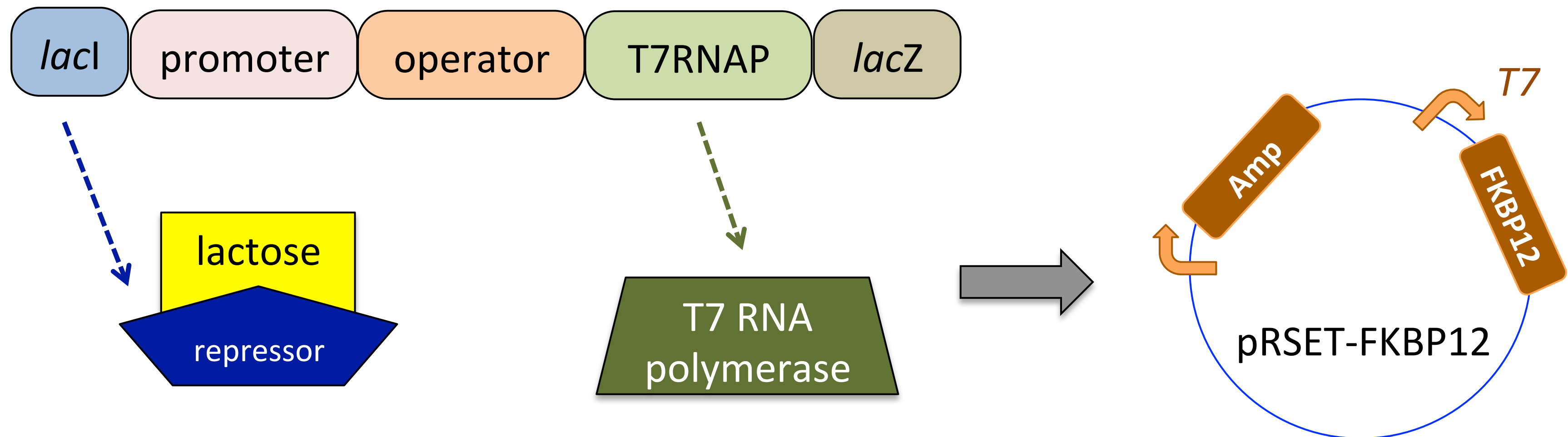
① in the pRSET plasmid, T7 promoter *on* only if T7RNAP present

② in BL21(DE3)pLysS, *lac*I => repressor binds control area => T7RNAP turned *off*

③ if lactose is present

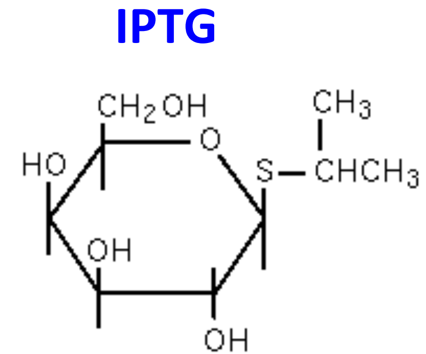
– lactose binds to repressor and makes it ___inactive___ , thus turning _on_ expression of T7RNAP

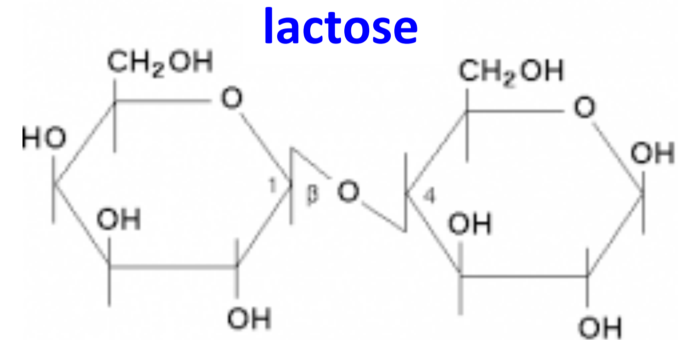
– with T7RNAP present, the T7 promoter is ___on___, and FKBP12 expressed

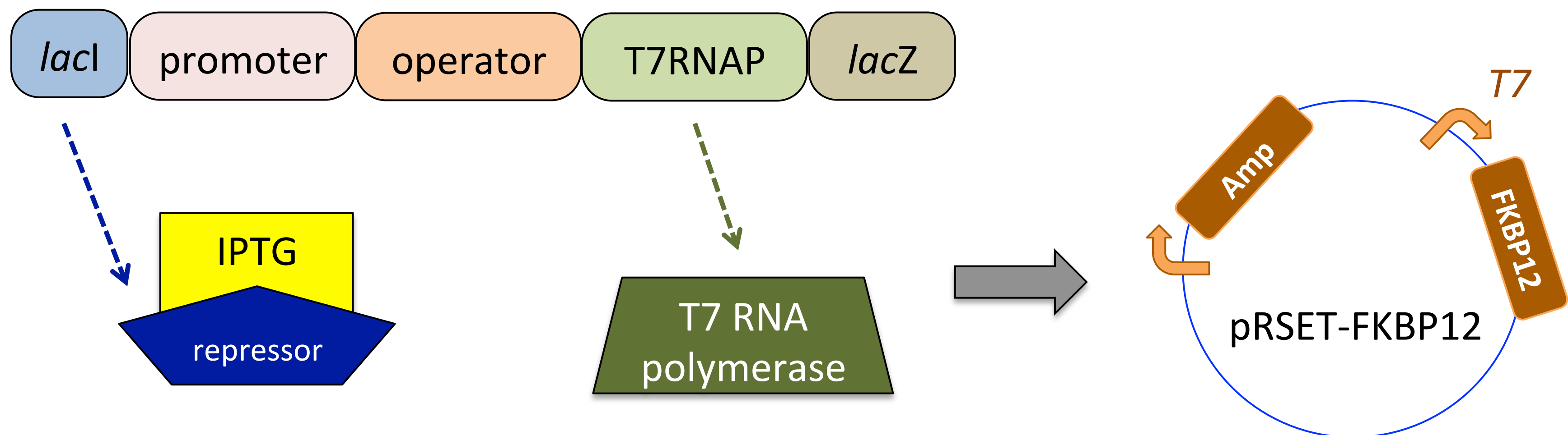


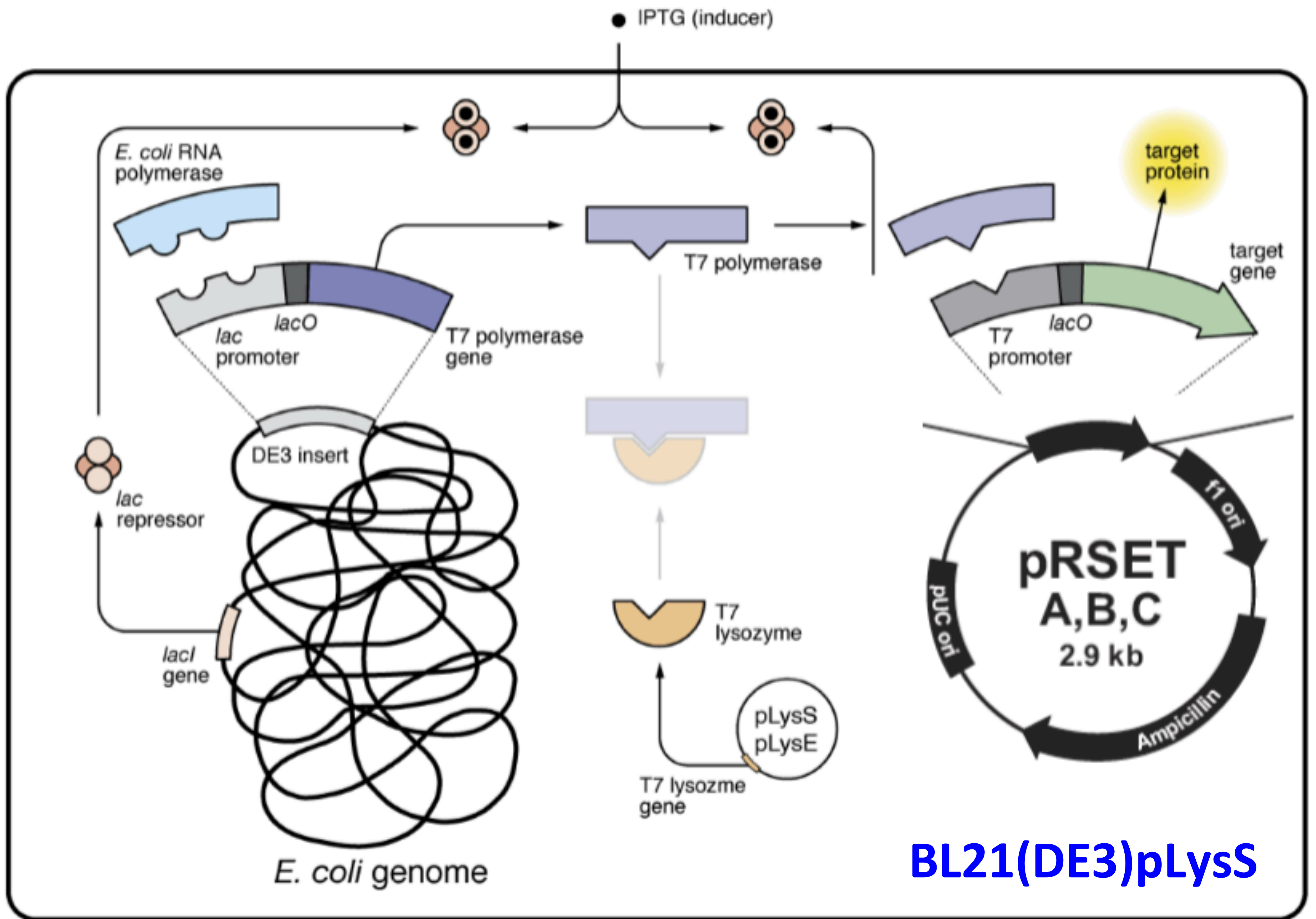
IPTG is a lactose analogue

- isopropyl β-D-1-thiogalactoside
- structural mimic of lactose
- unlike lactose, IPTG is not cleaved by β-galactosidase and so will not used by the cell
  ➔ [IPTG] constant

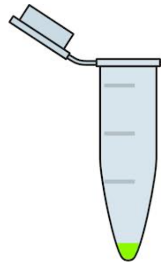




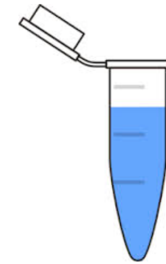



IPTG (inducer)
E. coli RNA polymerase
T7 polymerase
target protein
target gene
lac promoter
lacO
T7 polymerase gene
T7 promoter
lacO
DE3 insert
lac repressor
lacI gene
T7 lysozyme
pLysS
pLysE
T7 lysozme gene
f1 ori
pUC ori
pRSET A,B,C 2.9 kb
Ampicillin
E. coli genome
BL21(DE3)pLysS

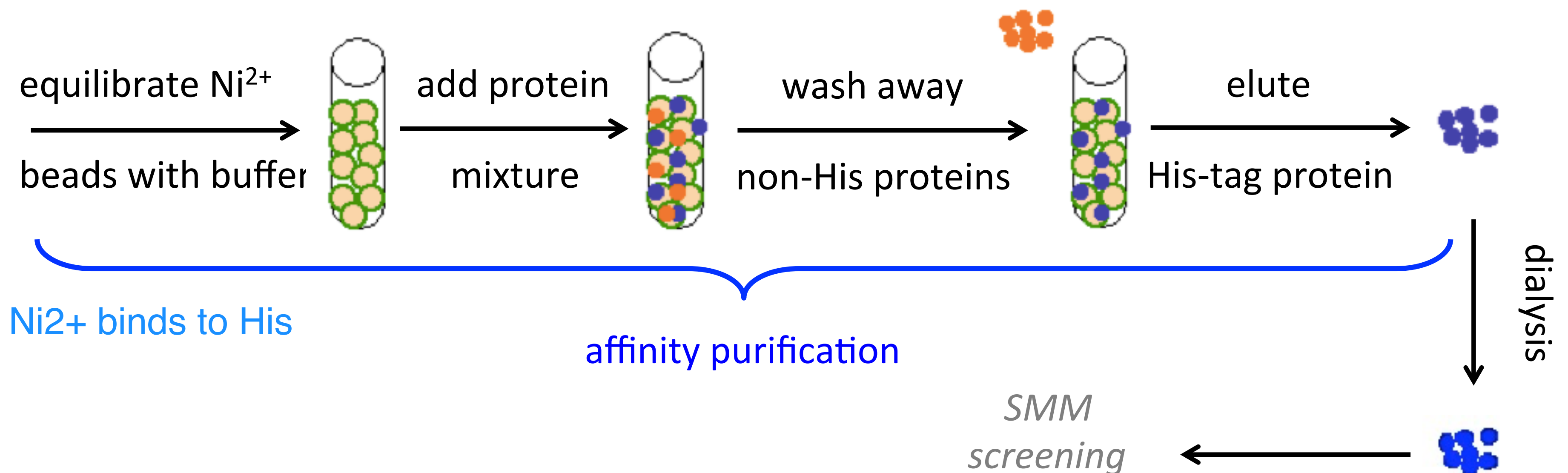
Protein purification: protocol overview



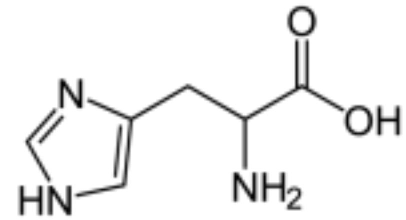
lyse (and extract supernatant)



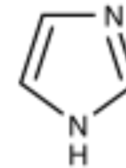
- protease inhibitor: AEBSF, helps keep proteins intact
- DNAase (deoxyribonuclease): chews up DNA
- Tris buffer: pH ~ 7
- salts: maintains osmotic pressure (ionic strength)
- lysozyme: damages E. coli cell wall
- DTT (dithiothreitol): reducing agent, reduces oxidative damage
- glycerol: stabilizer



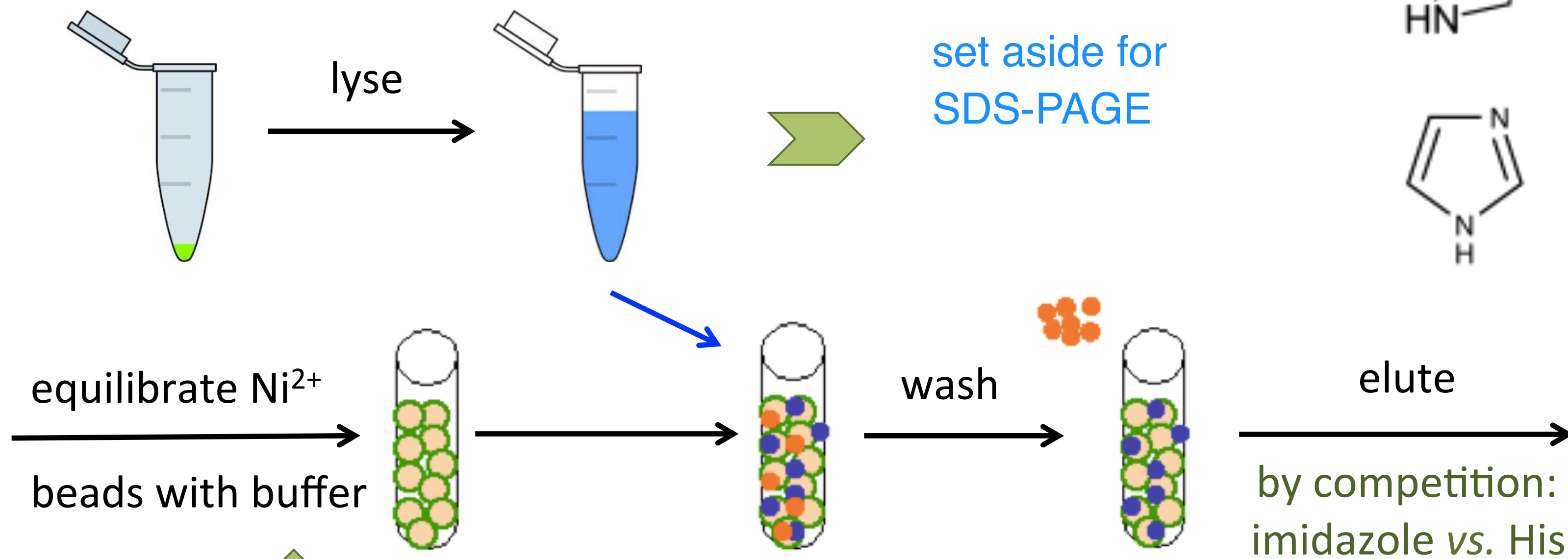
Protein purification: a few notes



histidine

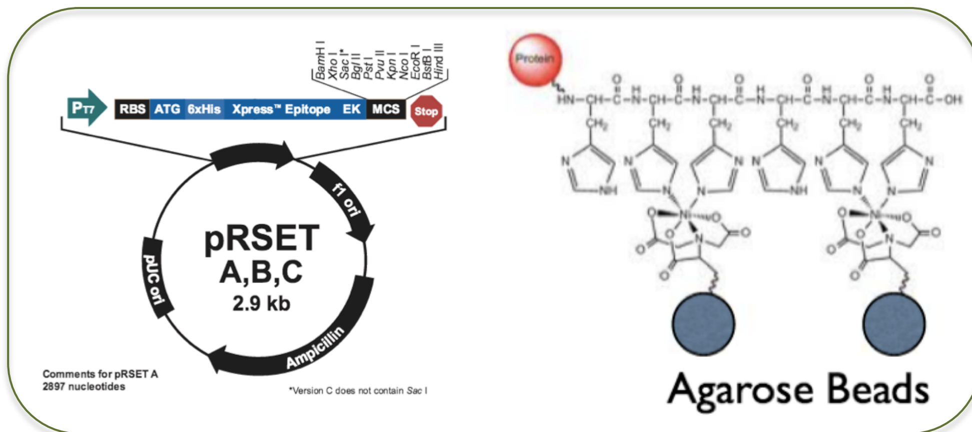


imidazole



His-tag

binds to metals



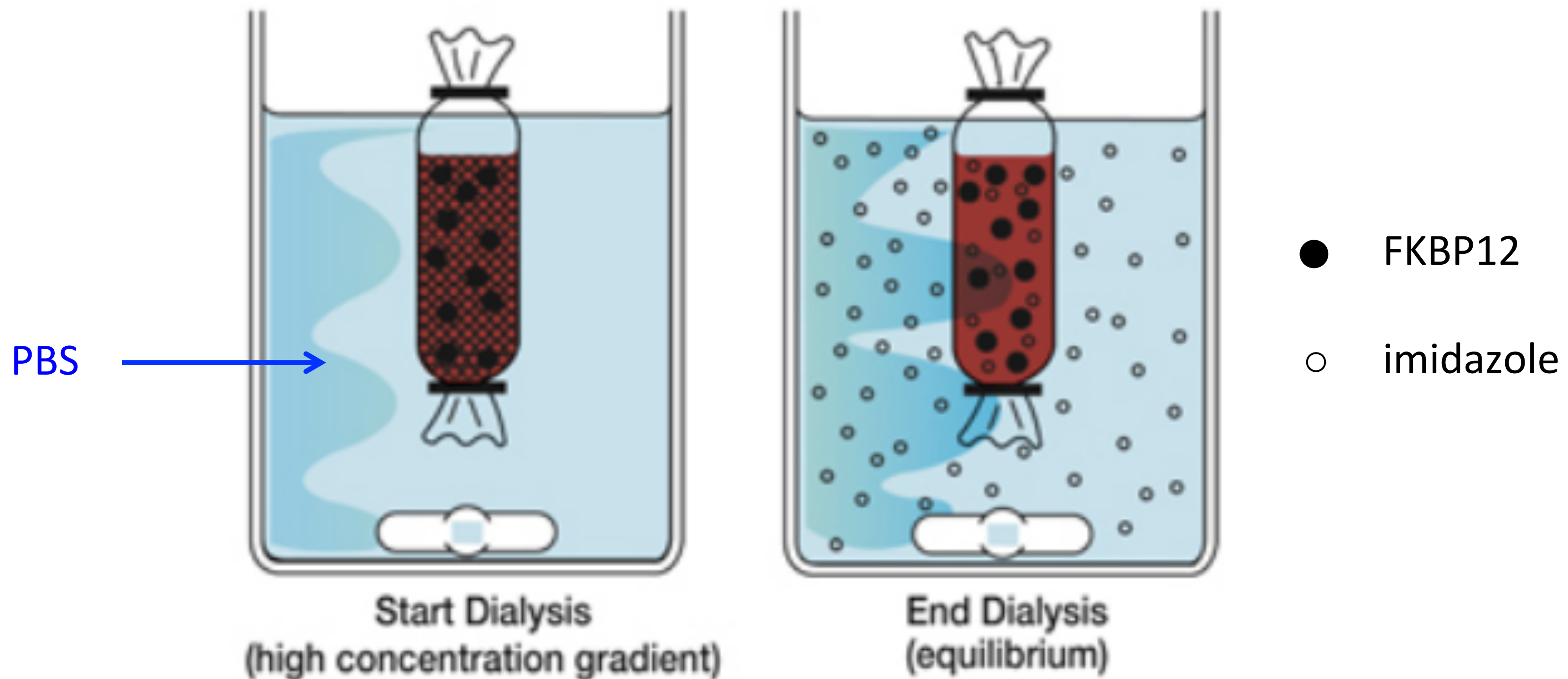
dialysis

imidazole can interfere with FKBP12-small molecule binding, so wash away imidazole

SMM ligand screening

Dialysis: separation based on size rejection

- semipermeable membrane of crosslinked polymers
- molecular weight cut-off (MWCO): solute size retained > 90%
- 2000 Da
  - FKBP12-6His ~ 15 kDa -- imidazole ~ 68 Da



Looking ahead

- In the lab today
  - Purify FKBP12
  - Orientation quiz (during down time)

- Homework due Thursday, M1D3
  - Schematic of M1 (+ title + caption) of protein purification
  - Before M1D4, visit Comm Lab
  - Quiz on M1D3